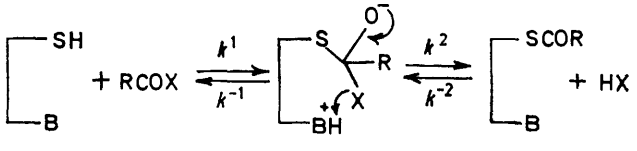
Proteolytic Enzymes. Papain-catalysed Hydrolysis of Specific Aryl Esters

By A. Williams,* E. C. Lucas, and A. R. Rimmer, University Chemical Laboratories, Canterbury, Kent

Aryl hippurates (I) and mesylglycinates (II) are hydrolysed in the presence of papain and have constant k_0 parameters of *ca.* 4 s^{-1} and 12 s^{-1} respectively at pH 6·00, 35° and 0·3M ionic strength. The low sensitivity of k_0/K_m to change in phenyl substituent [ρ = 0·47 and 0·78 for (II) and (I) respectively] is argued to arise from electrophilic assistance on the carbonyl oxygen of the ester during acylation but the involvement of imidazolium ion (of histidine-159) as the electrophile is discounted. Evidence is reported for the involvement of a *cationic* acid (pK_a *ca.* 4) in acylation and deacylation and a neutral acid (pK_a *ca.* 8) in acylation. 4-Nitrophenyl acetate (III) is a substrate of papain with a typical bell-shaped profile for k_0/K_m; the ester is shown to differ from specific substrates only in its electronic reactivity.

Lowe [1] recently reported that the rate constant for acylation of papain by hippuryl anilides obeys a Hammett relationship with negative ρ and interpreted the result as general acid catalysis of the departure of anilide from a tetrahedral intermediate. The overall rate constant $[k^1k^2/(k^{-1} + k^2)]$ involves the acid-catalysed step (k^2) and thus any electrophilic assistance on k^1, the addition step (which involves also the binding of enzyme and substrate), is obscured. Acylation of papain by phenyl esters should involve a rate-limiting addition step ($k^{-1} < k^2$) owing to the good leaving ability of the phenyl leaving group. Acylation of chymotrypsin by specific phenyl ester substrates is insensitive to the substituent on the phenyl (ρ ~0·5) [2] but early work on the acylation of papain by specific phenyl esters indicated a higher sensitivity (~1·2).[3] We



(1)

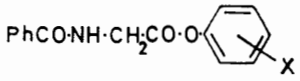
decided to reinvestigate the acylation of papain as the Hammett ρ values are based on a small number of substituents.[3] Our data are more extensive and the

[1] G. Lowe, *Phil. Trans.*, 1970, **257***B*, 237.

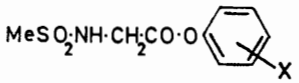
[2] (*a*) A. Williams, *Biochemistry*, 1970, **9**, 3383; (*b*) R. E. Williams and M. L. Bender, *Canad. J. Chem.*, 1971, **49**, 210.

[3] (*a*) G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 199; (*b*) J. F. Kirsch and M. Igelström, *Biochemistry*, 1966, **5**, 783.

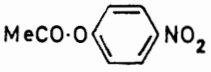
sensitivity value is thus more reliable. The esters studied here are:



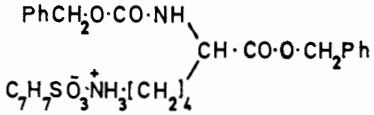
(I) a,b,c,f,j,l,o



(II) a-n

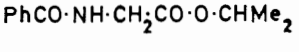


(III)

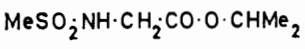


(S)-(IV)

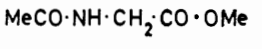
(V)



(VI)



(VII)



(VIII)

a; Phenyl
b; 4-Nitrophenyl
c; 3-Nitrophenyl
d; 4-Acetylphenyl
e; 4-Formylphenyl
f; 4-Chlorophenyl
g; 3-Methoxyphenyl
h; 4-Methoxyphenyl
i; 3-Fluorophenyl
j; 4-Fluorophenyl
k; 3-Methylphenyl
l; 4-Methylphenyl
m; 4-Hydroxyphenyl
n; 4-Benzyloxyphenyl
o; 4-Aminophenyl

EXPERIMENTAL

Materials.—Papain (E.C.3.4.4.10) was prepared from the solidified papaya sap by the method of Kimmel and Smith.[4] We improved the method by using mercaptoacetic acid as reducing agent instead of the more expensive L-cysteine. The sand is omitted in the grinding procedure and the initial extraction is allowed to proceed for 2½ h at room temperature. The Wallerstein Corporation provided a gift of dried papaya sap. 4-Aminophenylhippurate (Io) was prepared by hydrogenation of the 4-nitrophenyl ester from another study [2a] in ethyl acetate solution using palladium–charcoal catalyst. After uptake of the theoretical volume of hydrogen the reaction was stopped and the suspension was filtered. The filtrate was washed with saturated sodium hydrogen carbonate solution and water, and then dried ($CaSO_4$); light petroleum (b.p. 60—80°) was added to precipitate the ester.

4-Hydroxyphenyl mesylglycinate (IIm) was prepared from the 4-benzyloxyphenyl ester by the above procedure. Aryl esters of mesylglycine (II) were prepared by adding the acid chloride (1 equiv.) [5] to a solution or suspension of the phenol (1 equiv.) in dichloromethane at 5 °C; the solvent was removed under reduced pressure and the residue was ground in the presence of water until it solidified. The solid was filtered, washed, and recrystallised.

Isopropyl *N*-benzyloxycarbonylglycinate (V) was prepared by dissolving the acid chloride in the alcohol.

Benzyl *N*-α-benzyloxycarbonyl-L-lysinate (IV) was prepared by refluxing the acid, a slight excess of toluene-*p*-sulphonic acid and an excess of benzyl alcohol in benzene for 4 h using a Dean and Stark apparatus to remove water. The benzene was evaporated *in vacuo* and the residue was induced to crystallise with dry ether.

The preparations of isopropyl hippurate (VI) methyl acetylglycinate (VIII), isopropyl mesylglycinate (VII), and 4-nitrophenyl acetate (III) are described elsewhere.[5-7]

4-Nitrophenyl *N*-benzyloxycarbonylglycinate was bought from Sigma Chemical Company.

Structures of the substrates were confirmed by i.r. and n.m.r. spectroscopy and analytical and physical properties are collected in Table 1.

Acetonitrile was purified by the method of Lewis and Smyth [8] and analytical grade dioxan was passed down an alumina column immediately before use to remove peroxides. Other materials were of analytical reagent grade and deionised water was used throughout the investigation.

Methods.—Papain was activated by a modification of the method of Soejima [9] and papain solutions were assayed as previously described.[5,10] Papain used with 4-nitrophenyl acetate (III) studies was assayed with 4-nitrophenyl *N*-benzyloxycarbonylglycinate at pH 6·00; owing to the low K_m for this substrate the assays were easily carried out at saturation of the enzyme thus the rate of hydrolysis was $k_0 \times [E]$. The value of k_0 is that previously determined.[10] Rates of ester hydrolysis were measured with a pH-stat or at a suitable u.v. wavelength (Table 2) using a Unicam SP 800 spectrophotometer. The data were treated as before [5] and algorithms used in the computations (Elliott 4130 computer) are published.[11] The dependence of k_0 [see equation (2)] on pH for substrates (IV)—(VIII) and (IId) was obtained using $[S] \gg K_m$ thus initial rates were proportional only to enzyme concentration; k_0/K_m pH-profiles were determined using $[S] \ll K_m$ thus initial rates were proportional to both substrate and enzyme concentrations. Owing to the high K_m and low k_0 for 4-nitrophenyl acetate (III) the pH-profile of k_0/K_m was determined under conditions where $[S] \ll K_m$. By these means pH-profiles were obtained in a few hours thus eliminating errors in enzyme assay inherent in separate determinations. Each rate was repeated twice and the average taken; they are corrected where necessary in pH-stat experiments for product acid protonation at low pH values using pK_a values determined under identical conditions: acetylglycine, 3·65 (35 and 55° water) and 3·75 (35° in 10% dioxan); hippuric acid in 10% acetonitrile, 3·75 (35°) and 3·80 (55°); mesylglycine, 3·2 (35°) and 3·25 (55°); *N*-benzyloxycarbonylglycine in 10% acetonitrile, 3·8 (35°) and 3·9 (55°); *N*-α-benzyloxycarbonyl-L-lysine at 25°, 3·53 (H_2O), 3·62 (5% dioxan), and 3·7 (10% dioxan). Heats of ionisation for the groups on the enzyme were

[4] J. R. Kimmel and E. L. Smith, *J. Biol. Chem.*, 1954, **207**, 515.
[5] E. C. Lucas and A. Williams, *Biochemistry*, 1969, **8**, 5125.
[6] A. Williams, E. C. Lucas, A. R. Rimmer, and Mrs H. C. Hawkins, following paper.
[7] A. Williams and R. A. Naylor, *J. Chem. Soc.* (*B*), 1971, 1967.
[8] G. L. Lewis and C. P. Smyth, *J. Chem. Phys.*, 1939, **7**, 1085.
[9] M. Soejima and K. Shimura, *J. Biochem.* (*Tokyo*), 1961, **49**, 260.
[10] A. Williams and E. C. Lucas, *Analyt. Chem.*, 1970, **42**, 1491.
[11] A. Williams, 'Introduction to the Chemistry of Enzyme Action,' McGraw-Hill, London, 1969 (Appendix).

TABLE 1

Analytical and physical properties of substrates [a]

Substrate	M.p. (t/°C)	Found (%) C	H	N	Formula	Calc. (%) C	H	N
(Ij)	102—105	68·1	4·2	5·6	$C_{15}H_{12}FNO_3$	68·4	4·6	5·3
(Io)	133—136	66·8	5·2	10·6	$C_{15}H_{14}N_2O_3$	66·7	5·2	10·4
(IIa)	113—113·5	47·2	4·8	6·1	$C_9H_{11}NSO_4$	47·0	5·0	6·2
(IIb)	153—155	39·0	3·5	10·1	$C_9H_{10}N_2SO_6$	39·4	3·7	10·2
(IIc)	113—116	39·4	3·9	10·0	$C_9H_{10}N_2SO_6$	39·4	3·7	10·2
(IId)	142—143	48·6	4·9	5·2	$C_{11}H_{13}NSO_5$	48·7	5·0	5·2
(IIe)	114—116	46·1	4·3	5·6	$C_{10}H_{11}NSO_5$	46·7	4·3	5·5
(IIf)	162—163	41·1	3·8	5·3	$C_9H_{10}ClNSO_4$	41·5	4·1	5·4
(IIg)	116—118	46·2	5·2	5·2	$C_{10}H_{13}NSO_3$	46·3	5·0	5·4
(IIh)	112—113	46·5	5·0	5·5	$C_{10}H_{13}NSO_5$	46·3	5·0	5·4
(IIi)	117·5—119	43·4	4·1	5·7	$C_9H_{10}FNSO_4$	43·7	4·1	5·7
(IIj)	136—137	43·7	4·2	5·7	$C_9H_{10}FNSO_4$	43·7	4·1	5·7
(IIk)	145—146	49·4	5·4	5·8	$C_{10}H_{13}NSO_4$	49·4	5·4	5·8
(IIl)	115—117	49·4	5·2	5·6	$C_{10}H_{13}NSO_4$	49·4	5·4	5·8
(IIm)	156—157	44·1	4·8	5·5	$C_9H_{11}NSO_5$	44·1	4·5	5·7
(IIn)	157—159	57·2	4·9	4·2	$C_{16}H_{17}NSO_5$	57·3	5·1	4·2
(IV)	113—115 [b]	62·2	6·4	5·0	$C_{23}H_{34}N_2SO_7$	62·0	6·3	5·2
(V)	53—55	62·8	6·9	5·6	$C_{13}H_{17}NO_4$	62·2	6·8	5·6

[a] Microanalyses performed on a Hewlett–Packard model-185 analyser by Mrs. M. J. Clark. Melting points were recorded using a Kofler Thermospan instrument. [b] Lit. m.p. 111—112 °C (ref. 14).

calculated from the apparent pK_a values using the equation $H_i = -2{\cdot}303\ RT^2\ \Delta\ pK_a/\Delta T$.

TABLE 2

Spectral data for hydrolysis of aryl esters [a]

Substrate	λ/nm	Molar extinction coefficient
(Io)	310	710
(Ij)	280	2070
(IIm)	290	1460
(IIf)	281	1380
(IIb)	325	7290
(IId)	295	7890
(IIn)	285	2180

[a] Results apply to pH 6·00, 0·3M-ionic strength, 35°, and 10% (v/v) acetonitrile.

RESULTS

The rates of papain-catalysed hydrolysis for the substrates studied here obeyed Michaelis–Menten kinetics

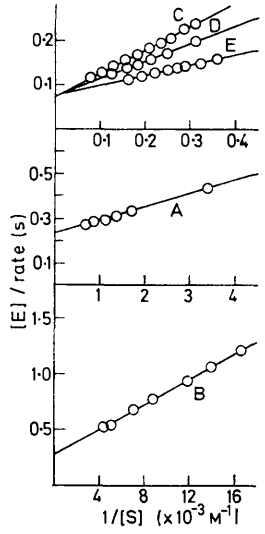


FIGURE 1 Typical kinetic results for substrates A, 4-aminophenyl hippurate (Io); B, 4-fluorophenyl hippurate (Ij); C, 4-methoxyphenyl mesylglycinate (IIh); D, 3-methoxyphenyl mesylglycinate (IIg); E, 3-nitrophenyl mesylglycinate (IIc); lines are theoretical [equation (2), Tables 3 and 4]

[Figure 1 and equation (2)]. 4-Benzyloxyphenyl mesylglycinate was too insoluble to obtain accurate individual

$$\text{rate} = k_0[E][S]/([S] + K_m) \qquad (2)$$

parameters and k_0/K_m was determined at low substrate

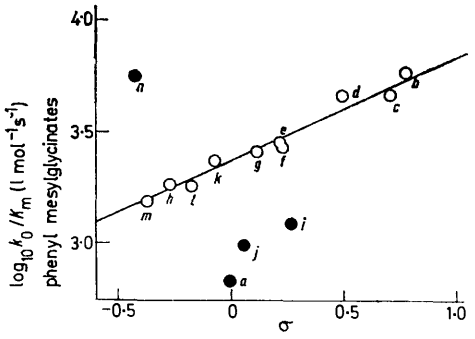


FIGURE 2 Dependence on σ of k_0/K_m for aryl mesylglycinates (II). Data from Table 3, σ values from G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75, line is theoretical; ●, points omitted from correlation

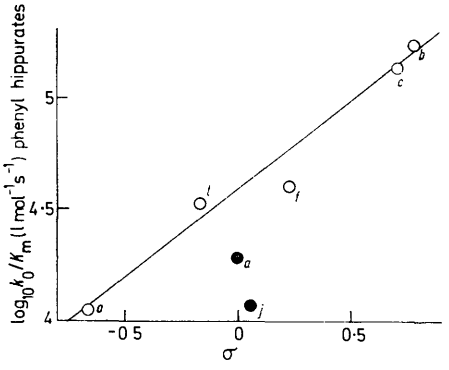


FIGURE 3 Dependence on σ of k_0/K_m for aryl hippurates (I). Data from Table 4, σ values from G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75, ●, points omitted from correlation

concentration where rate = $k_0/K_m[E][S]$. The esters of mesylglycine and hippuric acid, (I) and (II), have essentially

similar values for k_0 (12 and 4 s^{-1} respectively) but the values for k_0/K_m obey Hammett relationships (Figures 2 and 3, Tables 3 and 4). The sensitivity to σ is computed to be

TABLE 3

Kinetic data for mesylglycinate esters (II) [a]

Ester	k_0/s^{-1}	$K_m/M(\times 10^3)$	$(k_0/K_m)/l\ mol^{-1}$ $(\times 10^{-3})$
(IIm) [c]	13·6 ± 0·6	8·9 ± 0·5	1·53 ± 0·11
(IIh)	13·7 ± 0·2	7·48 ± 0·23	1·83 ± 0·03
(III)	15·0 ± 0·7	8·32 ± 0·62	1·80 ± 0·15
(IIk)	13·8 ± 0·6	5·85 ± 0·53	2·36 ± 0·25
(IIa)	10·8 ± 0·3	16·2 ± 0·6	0·682 ± 0·028
(IIj)	11·0 ± 0·3	11·3 ± 0·5	0·978 ± 0·04
(IIg)	13·3 ± 0·3	5·14 ± 0·28	2·59 ± 0·14
(IIe)	13·3 ± 0·7	4·73 ± 0·62	2·81 ± 0·37
(IIf) [c]	11·1 ± 1·0	4·15 ± 0·43	2·68 ± 0·36
(IIi)	13·2 ± 0·3	10·5 ± 0·4	1·24 ± 0·08
(IId)	12·3 ± 0·3	2·69 ± 0·15	4·57 ± 0·27
(IIc)	13·3 ± 0·4	2·92 ± 0·26	4·56 ± 0·36
(IIb) [c]	11·1 ± 0·9	1·93 ± 0·20	5·75 ± 0·75
(IIn) [c]	—	—	5·66 [b]

[a] pH 6·00, 35°, 0·3M ionic strength, 10% CH_3CN, results obtained *via* pH-stat except where stated. [b] Too insoluble to obtain accurate values of k_0 and K_m. Ratio k_0/K_m obtained from pseudo first-order kinetics at $[S] < K_m$. [c] Results obtained *via* spectrophotometric method.

TABLE 4

Kinetic data for hippurate substrates (I) [d]

Ester	k_0/s^{-1}	$K_m/M(\times 10^4)$	$(k_0/K_m)/l\ mol^{-1}$ s^{-1} $(\times 10^4)$
(Io) [a]	4·33 ± 0·04	2·62 ± 0·09	1·66 ± 0·06
(Il) [b]	4·69 ± 0·08	0·952 ± 0·008	4·94 ± 0·03
(Ia) [c]	4·46 ± 0·52	1·7 ± 0·15	2·81 ± 0·48
(If) [c]	5·85 ± 1·03	1·01 ± 0·23	5·84 ± 0·42
(Ij) [a]	3·53 ± 0·18	2·04 ± 0·20	1·73 ± 0·19
(Ic) [c]	3·65 ± 0·04	0·183 ± 0·006	19·9 ± 0·63
(Ib) [c]	3·41 ± 0·39	0·140 ± 0·034	25·3 ± 4·2

[a] This work. [b] Parameters from ref. 3*a* corrected using $k_0 = 4{\cdot}02\ s^{-1}$ for methyl hippurate assay substrate instead of 2·7 s^{-1}. [c] Parameters from A. Williams, D.Phil. Thesis, University of Oxford, 1964; corrected as in footnote *b*. [d] pH 6·00, 0·3M-ionic strength, 35°.

0·47 ($r = 0{\cdot}985$) for the mesylglycinates when substituents (IIn, a, i, j) are omitted and that for hippurates is 0·78 ($r = 0{\cdot}951$) when (Ia, j) are omitted. The higher ρ value

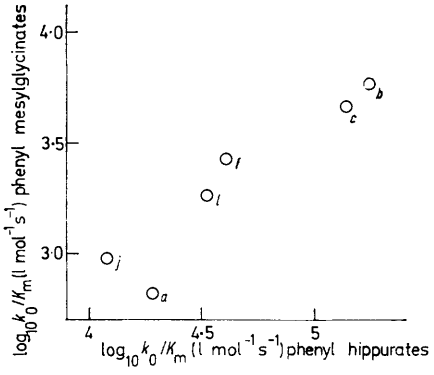


FIGURE 4 Linear free-energy relationship between k_0/K_m for aryl mesylglycinates (II) and aryl hippurates (I)

previously observed for *N*-benzyloxycarbonylglycine and hippurate aryl esters [3] is due to inclusion in the correlation of the phenyl substituent in plots with total numbers of points 3 and 4 respectively. The reason for neglecting deviant points in calculating the Hammett sensitivity is justified later but Figure 4 demonstrates a linear free-energy relationship between k_0/K_m for hippurates and mesylglycinates including the deviants.

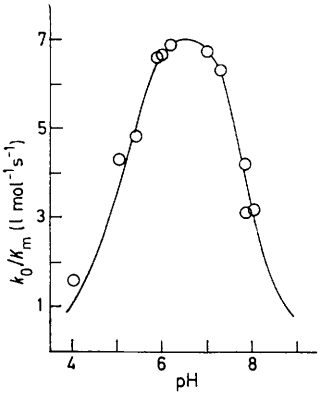


FIGURE 5 pH-Dependence of k_0/K_m for 4-nitrophenyl acetate. Data from Table 5, line theoretical for $pK_{a1} = 5$, $pK_{a2} = 8$

4-Nitrophenyl acetate hydrolysed with papain according to equation (2) but K_m was much higher than for specific substrates accounting for previous authors' inability to observe saturation.[12,13] The pH-profile of k_0/K_m (Table 5) is bell-shaped and illustrated in Figure 5; the pK_a values are similar to those for specific substrates.

TABLE 5

Hydrolysis of 4-nitrophenyl acetate-catalysed by papain [a]

pH	Buffer	$(k_0/K_m)/l\ mol^{-1}\ s^{-1}$ [c]	Δ 340 nm
4·35	Acetate	1·41 ± 0·1	5062
5·12	Acetate	4·16 ± 0·4	5062
5·50	Acetate	4·69 ± 0·3	5062
5·90	Phosphate	6·45 ± 0·5	4961
6·00 [b]	Phosphate	6·50 ± 0·7	4910
6·35	Phosphate	6·74 ± 0·6	4708
7·00	Phosphate	6·62 ± 0·5	4050
7·30	Tris	6·21 ± 0·4	3746
7·90	Tris	4·1 ± 0·4	3341
8·00	Phosphate	3·05 ± 0·4	3290
8·15	Tris	3·11 ± 0·3	3290

[a] 25°, 0·1M-ionic strength, $[E] = 0{\cdot}51 \cdot 10^{-5}M$, $[S] = 2—10 \cdot 10^{-2}M$; $k_0 = 7{\cdot}6 \pm 0{\cdot}5 \cdot 10^{-2}\ s^{-1}$; the value of k_0/K_m at pH 6·00 agrees with that obtained by de Jersey (ref. 12) from pseudo-first-order kinetics (6·12 l mol^{-1} s^{-1}, $[S] = 0{\cdot}2—1{\cdot}10^{-2}M$). [c] Corrected for spontaneous hydrolysis by buffer.

The dependence of Michaelis–Menten parameters on dioxan concentration and on temperature was determined using substrates (IV)—(IX), (IId) where it was possible to determine k_2 or k_3 unambiguously [see equation (3) and reference 5]. The results are illustrated in Figures 6 and 7 and collected in Table 6.

DISCUSSION

Much evidence points to equation (3) as the stoicheiometric mechanism for papain-catalysed ester hydrolysis.[1,3,14] Cysteine-25 * of the enzyme acts as the

* The numbering scheme employed here is that of S. S. Husain and G. Lowe, *Biochem. J.*, 1969, **114**, 279.

[12] J. de Jersey, *Biochemistry*, 1970, **9**, 1761.
[13] L. J. Brubacher, Thesis, Northwestern University, 1966.
[14] (*a*) M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, 1966, **88**, 5880; (*b*) p. 5871.

TABLE 6

Effect of dioxan and temperature on apparent ionisation constants

Substrate	t/°C	pK_{a1}	H_1/kcal mol^{-1} [a]	pK_{a2}	H_1/kcal mol^{-1} [a]
		(Values for k_2)			
(VI)	35	—		8·4	
	55	4·2	—	8·12	5·9
(V)	35	4·43		8·54	
	55	4·20	5·0	8·22	7·0
(VII)	35	4·51		8·54	
	55	4·25	5·7	8·20	7·4
(VIII)	35	4·50		8·3	
	55	4·28	4·8	—	—
	35 [b]	3·90 [b]			
(IId)	35	3·9		8·2	
	35 [b]	3·8 [b]		8·8 [b]	
		(Values for k_3)			
(IV)	25	3·43			
	25 [c]	3·10 [c]			
	25 [b]	2·70 [b]			

[a] 35°. [b] 10% Dioxan. [c] 5% Dioxan.

nucleophile and the imidazolyl moiety of histidine-159 is involved in both k_2 and k_3 as a general base. We

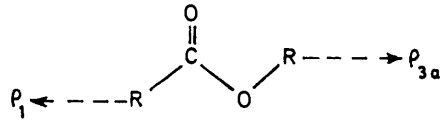
$$\mathrm{RCOX} + \mathrm{ESH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ESH} \cdots \mathrm{RCOX} \xrightarrow{k_2} \mathrm{ESCOR} + \mathrm{HX} \quad (3)$$

$$\mathrm{ESCOR} \xrightarrow{k_3} \mathrm{ESH} + \mathrm{RCO_2H}$$

have recently shown [5] that some substrates of papain have $k_{-1} \gg k_2$ thus $k_0/K_m = k_1k_2/k_{-1}$; k_0/K_m is the bimolecular rate constant for reaction of *free* substrate with *free* enzyme to give acyl-enzyme. There is a good correlation between log k_0/K_m and σ for aryl mesylglycinates (II) when certain substituents are omitted. Acceleration of the 4-benzyloxyphenyl ester (IIn) could be due to an extra binding of the leaving group with the active centre at the ρ_{3a} site.[6] Rate depressions for phenyl, 3-fluorophenyl, and 4-fluorophenyl esters (IIa, i, j) could be caused by these moieties (which are essentially of the same size) taking up more degrees of freedom during acylation than larger substituted groups. The ρ_{3a} site accommodating the leaving phenol is lipophilic (see ref. 6) and could thus be sterically crowded. Omitting these abnormal substituents from the hippurate correlation (Figure 3) yields a low ρ value also.

Kirsch [3a] reported rate constants for reaction of mercaptoethanol thioanion with substituted phenyl *N*-benzyloxycarbonylglycinates and we calculate from his work a Hammett ρ value of *ca.* 2; Lowe reports a value of 1·7 for reaction of the anion $\overset{+}{\mathrm{N}}\mathrm{H_3CH(CO_2^-)CH_2S^-}$ with phenyl acetates [3a] and we have found [15] a value of 2·3 for phenyl acetates with mercaptoacetate and 1·7 for phenyl mesylglycinates with $\overset{+}{\mathrm{N}}\mathrm{H_3CH(CO_2^-)CH_2S^-}$. A strong argument [5] has been presented that the nucleophile of papain is $-SH$ rather than $-S^+$; the un-ionised thiol should yield sensitivity to σ greater than that for the thiolate nucleophile because it is the weaker and bond formation between carbonyl carbon and sulphur should thus be more advanced in the transition state. The Taft ρ^* for reaction of water with ethyl esters is 3·5 (ref. 16*a*) as opposed to the sensitivity of 2·5 (ref. 16*b*) for reaction of hydroxide ion. General base catalysed hydrolysis of substituted phenyl diphenylphosphinates has a Hammett ρ of 2·88 as opposed to one of 1·55 for the hydroxide reaction.[7] No variation in sensitivity is expected between intramolecular reaction as in the enzyme and intermolecular reaction as in model systems.[17]

The low sensitivity is not due to proton donation in the k_2 step by a general acid as this rate constant is not included in k_0/K_m for phenyl esters. Reactivity arguments cannot explain the low sensitivity because although mesylglycine and hippurate esters are considerably more reactive than acetate esters (see following paper and ref. 18) sensitivities to change of aryl leaving group in attack by thiolate anion are not very different. Electrophilic participation provides the simplest explanation of the low sensitivity and we suggest this occurs on the carbonyl oxygen. Electrophilic participation probably does not occur at the leaving heteroatom in the formation of the tetrahedral adduct because the acid that performs this function for poor leaving groups in the k^2 step is involved as a base in the k^1 step. Binding sites (ρ_1 and ρ_{3a}) at the enzyme-active site [6] could activate the



(IX)

ester bond by distortion. However, the sensitivity for hippurate and mesylglycinates is similar despite the 50-fold difference in reactivity caused by a stronger ρ_1 binding.[6] Sensitivities to leaving group appear constant over a wide range of reactivity (Table 7) thus if the enzyme distorts the ester bond this is not reflected in a change of sensitivity to leaving group.

TABLE 7

Comparison of sensitivity of k_0/K_m to leaving group with reactivity [f]

	(k_0/K_m)/l mol^{-1} s^{-1}		
Acid	4NP	ME	BE
Mesylglycine	5·75 . 10^3 [d]	19 [e]	1·84 . 10^3 [b]
Hippuric acid	2·53 . 10^5 [c]	246 [b]	5·5 . 10^3 [h]
Z-Glycine	2·87 . 10^5 [c]	382 [a]	—
Acetylglycine	2·39 . 10^3 [b]	2·06 [b]	207 [b]
Acid	4NP/ME	BE/ME	
Mesylglycine	3·02 . 10^2	97	
Hippuric acid	10·3 . 10^2	22·4 [g]	
Z-Glycine	7·5 . 10^2	—	
Acetylglycine	11·6 . 10^2	101	

[a] Data from ref. 3*b*. [b] Data from ref. 5. [c] See Table 4. [d] See Table 3. [e] This work. [f] Abbreviations: Z, benzyloxycarbonyl; 4NP, 4-nitrophenyl ester; ME, methyl ester; BE, benzyl ester. [g] The effect of 20% CH_3CN on the ρ_3 site is unknown. [h] Data from ref. 6.

[15] A. Williams and E. C. Lucas, unpublished observations.

[16] (*a*) W. P. Jencks and J. Carriulo, *J. Amer. Chem. Soc.*, 1961, **83**, 1743; (*b*) R. W. Taft, 'Steric Effects in Organic Chemistry,' ed. M. S. Newman, John Wiley, New York, 1956, 556.

[17] T. C. Bruice and S. J. Benkovic, *J. Amer. Chem. Soc.*, 1963, **85**, 1.

The hydrolysis of 4-nitrophenyl acetate by papain has a bell-shaped pH-profile for k_0/K_m and hence probably hydrolyses *via* a mechanism similar to that for specific

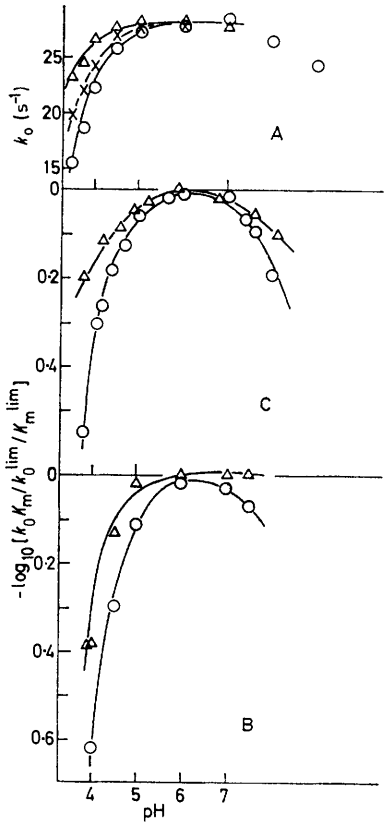
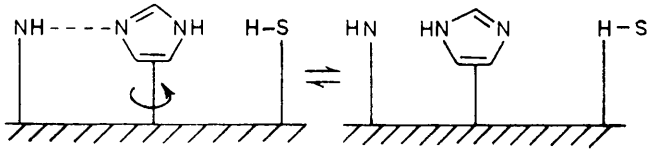


FIGURE 6 Effect of dioxan on pK_a values for: A, benzyl N-α-benzyloxycarbonyl-L-lysinate (IV) (k_3); B, methyl acetylglycinate (VIII) (k_3); C, 4-acetylphenyl mesylglycinate (IId) (k_2): ○ = 0% dioxan, × = 5% dioxan, △ = 10% dioxan

substrates. Since specific benzyl esters are 100-fold more reactive than methyl esters the binding constant for the benzyl portion with the enzyme, provided the methyl group has no affinity, is 10^{-2}M which is close to the K_m value for 4-nitrophenyl acetate; we propose that the nitrophenyl group binds at the same site as the benzyl group (ρ_{3a}).[6] The low reactivity of ethyl acetate to hydroxide ion ($6{\cdot}21 \times 10^{-3}$ l mol^{-1} s^{-1} at 25°) [18c] compared with ethyl mesylglycinate ($3{\cdot}6$ l mol^{-1} s^{-1} at 25°) [18a] is reflected in the comparison of k_0/K_m for the 4-nitrophenyl esters.

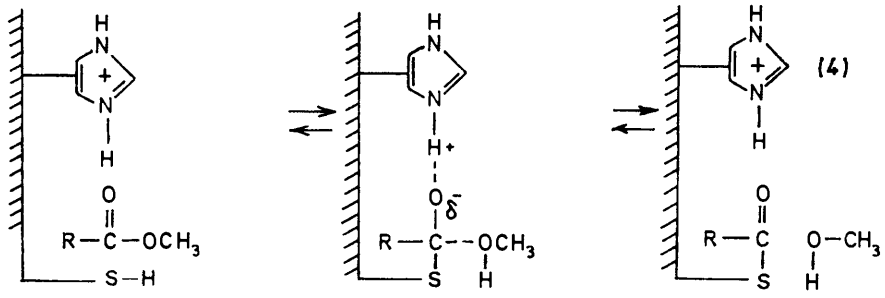
The effect of dioxan on the pK_a values for k_2 and k_3 (Table 6 and Figure 6) is in accord with a cationic acid of pK_a *ca.* 4 for k_2 and k_3 which becomes more acidic as the dioxan concentration increases [1] and a neutral acid for k_2 of pK_a *ca.* 8 which becomes less acidic as dioxan concentration goes up. Heats of ionisation are in accord with an imidazolyl and a thiol group. The lowering of the imidazolyl pK_a from a normal value of 7 to *ca.* 4 could be due to a lipophilic microscopic medium. Recently we proposed hydrogen bonding from NH of the amide of asparagine-175 to the imidazolyl tertiary nitrogen as an alternative explanation of the low pK_a but a major objection to this scheme is that the base must free itself from the hydrogen bond interaction and the C_β–C_γ bond must rotate prior to general base action (X). This is not in accord with the relatively rigid structure observed by *X*-ray crystallography [19] or with the observation of only one relatively narrow C-2-imidazolyl proton in the n.m.r. spectrum due



(X)

presumably to His-81 (personal communication from Dr. G. Lowe).

A recently proposed mechanism [equation (4)] involves imidazolium ion as an electrophile acting on carbonyl oxygen [19c,20] and could therefore predict a low Hammett ρ for acylation by phenyl esters. The absence of an apparent ionisation in k_3 corresponding to the imidazolyl group is explained by an abnormally high pK_a due to (*a*) the carboxylate anion (aspartate-



[18] (*a*) R. P. Bell and B. A. W. Coller, *Trans. Faraday Soc.*, 1964, **60**, 1087; (*b*) M. L. Bender, F. J. Kézdy, and C. R. Gunter, *J. Amer. Chem. Soc.*, 1964, **86**, 3714; (*c*) National Bureau of Standards Circular 510, 'Tables of Chemical Kinetics.'

[19] (*a*) J. Drenth, J. N. Jansonius, R. Koekoek, H. M. Swen, and B. G. Wolthers, *Nature*, 1968, **218**, 929; (*b*) J. Drenth, J. N. Jansonius, R. Koekoek, L. A. A. Sluyterman, and B. G. Wolthers, *Phil. Trans.*, 1970, **257***B*, 231; (*c*) B. G. Wolthers, J. Drenth, J. N. Jansonius, R. Koekoek, and H. M. Swen, 'Structure–Function Relationships of Proteolytic Enzymes,' eds. P. Desnuelle, H. Neurath, and M. Ottesen, Munksgaard, Copenhagen, 1970, 272.

[20] L. A. A. Sluyterman and B. G. Wolthers, *K. ned. Akad. Wetensch. Proc. Ser. B.*, 1969, **72**, 14.

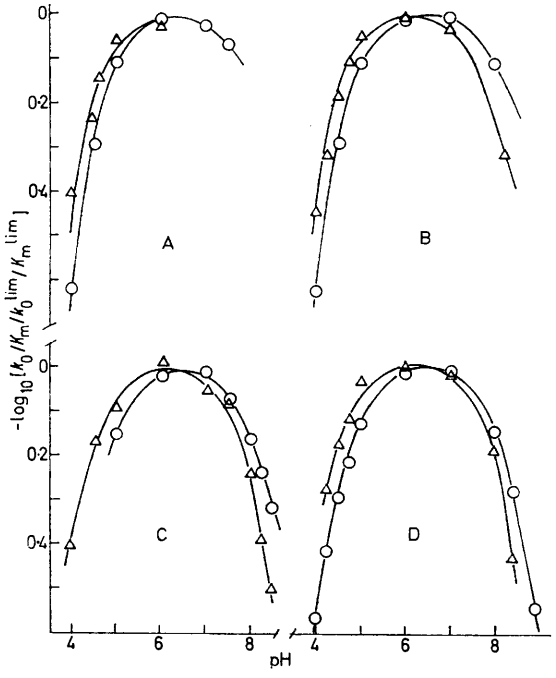


FIGURE 7 Effect of temperature on pK_a values for k_2: A, methylacetylglycinate (VIII); B, isopropyl mesylglycinate (VII); C, isopropyl hippurate (VI); D, isopropyl benzyloxycarbonylglycinate (V): ○ = 35°, △ = 55°

158) at a distance of 0·6 nm causing an increase in 1 pK_a unit (*b*) a hydrogen bond between side-chain oxygen of asparagine-175 and side-chain NH of histidine-159. It was also suggested that the lipophilic surroundings of the latter bond cause an increase of about 2 pK_a units. Since the imidazolyl of histidine-159 is surrounded by lipophiles we would expect a decrease in pK_a[1,21] and hydrogen bonding of a carboxylate anion with imidazolyl NH in a lipophilic medium [22] causes essentially no alteration in pK_a. The lower limb of the bell-shaped pH-profile was ascribed to substrate binding being affected by the carboxy-group [20] of aspartate-158. This is not in accord with the thermodynamic and solvent-composition studies of this report and previous work [5] indicates the independence of substrate binding on a low pK_a.

There is little chemical precedent for the action of imidazolium ion as an electrophile in substitution reactions at a carbonyl centre nor for the four-centre process involving sulphur, hydrogen, oxygen, and carbon in equation (4).

[1/1412 *Received*, 10*th August*, 1971]

[21] (*a*) M. Mandel, *Ind. chem. belge.*, 1958, **23**, 721; (*b*) D. Findlay, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, 1962, **85**, 139.

[22] (*a*) D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature*, 1969, **221**, 337; (*b*) F. J. Kézdy, A. Thomson, and M. L. Bender, *J. Amer. Chem. Soc.*, 1967, **89**, 1004.